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Reversed-phase chromatographic behaviour of β-endorphin: evidence of conformational change

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ABSTRACT

The effect of alteration in isocratic mobile phase constituents, composition of sample solution, flow-rate and column temperature on the reversed-phase chromatographic behaviour of β-endorphin was investigated. β-Endorphin was shown to be particularly sensitive to the concentration of organic modifier within the mobile phase. The relative contact area of β-endorphin was demonstrated to be less than that of the much smaller molecule, γ-endorphin, indicating that β-endorphin is in a folded form under the mobile phase conditions utilised. Buffer molarity and pH were implicated in the conformational transition of β-endorphin. In addition, the micro-environment of β-endorphin prior to its injection onto the high-performance liquid chromatographic (HPLC) column is crucial to its chromatographic behaviour. Manipulation of the sample solvent environment produced reversible conformational modifications ultimately resulting in asymmetric and even split peaks. This phenomenon was more clearly seen when altering HPLC flow-rate. Elevation of HPLC column temperature provided additional evidence of structural change in β-endorphin, with further conformational forms of this molecule being observed at higher temperatures. This work suggests that the chromatography of β-endorphin involves a complex mechanism of separation which cannot be adequately explained by the two-state model of kinetic processes.

INTRODUCTION

High-performance liquid chromatography (HPLC) has rapidly attained prominence as a powerful separation mode for proteins and peptides. It is perhaps not surprising therefore that such an effective method of separation has been utilised for β-endorphin prior to analysis by radioimmunoassay. Numerous reversed-phase HPLC systems now exist for β-endorphin and employ a variety of solvent conditions [1–6]. Unfortunately, however, little is known of the chromatographic interactions of β-endorphin and the corresponding behaviour of this molecule during elution.

It has been shown that chromatographic conditions, particularly the stationary phase, can affect hydrophobic interactions that normally stabilise native protein conformations [7]. This can lead to proteins undergoing slow dynamic interconversion resulting in broad asymmetrical peaks and even multiple peaks, which correspond to the native and denatured forms, if the kinetic processes of conformational change are slow or irreversible during elution [8]. This phenomenon has been observed with a variety of proteins such as papain [9], ribonuclease A [10,11], lysozymes [12] and myoglobin [12]. Anomalous band broadening of β-endorphin has been observed during size-exclusion chromatography in the presence of dodecyl sulphate [13] and with reversed-phase HPLC [14,15], demonstrating the propensity of β-endorphin to change structure during separation.

It is now well documented that the conformational stability of β-endorphin is affected by changes in solvent environment. In water β-endorphin is almost devoid of secondary structure [16–21], however, methanol [18,21,22] and trifluoroethanol [16,17,23,24] have been shown to induce conformational transition. Helicity is augmented by increasing the percentage of organic solvent, yielding 50 and 60% α-helix at 90% methanol and 100% trifluoroethanol, respectively. Similar promotion of helix

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formation has been reported in the presence of sodium dodecyl sulphate [21,22] and various lipids [25]. In general, much higher concentrations of organic solvents are required to achieve the same effect as a surfactant in inducing an ordered structure. The occurence of helixing has, however, been noted at concentrations as low as 10% methanol and trifluoroethanol [22]. Thus it might be postulated that both a mobile phase containing organic solvent plus the influence exerted by a hydrophobic HPLC stationary phase may contribute to conformational change of β-endorphin.

Although it might be hypothesises that β-endorphin undergoes conformational change during HPLC, the only data available is from gradient elution studies [14,15]. This paper therefore investigates the chromatographic behaviour of β-endorphin utilising an isocratic system and the consequence of varying mobile phase constituents such as the percent of organic modifier, pH and buffer molarity and in addition, the effect of altering the solvent composition of the sample solution.

Investigations to characterise conformational change in proteins caused by reversed-phase HPLC have been undertaken using elevated column temperature and increased flow-rates [10,26,27]. Temperature enhances denaturation, while changes in flow-rate will allow alternative conformations to be eluted, provided that the rate of change is of the same order as the retention time. These methodologies were thus employed here in an attempt to further characterise the chromatographic behaviour of β-endorphin.

EXPERIMENTAL

Materials

Purified β-endorphin (human sequence) was purchased from Sigma (Dorset, UK) as was α-endorphin, γ-endorphin, Met-enkephalin and Leu-enkephalin. Acetonitrile (HPLC-S grade) was obtained from Rathburn (Scotland, UK), ammonium acetate (HPLC grade) from Fisons (Loughborough, UK) and orthophosphoric acid (Aristar) and potassium dihydrogenphosphate (Aristar) from BDH (Dorset, UK). High-purity water was produced by double destilling, de-ionising and filtering through 0.45-μm nylon membrane filter (Gelman Sciences, Northampton, UK).

Equipment and operating conditions

All chromatographic experiments were performed using a SP8750 organiser and SP8770 pump (Spectra-Physics, Herts., UK) with a Rheodyne injection valve, coupled with a single-pen chart recorder. Detection was obtained by a UV–VIS detector (LC871 Pye, Cambridge, UK) at 210 and 275 nm or electrochemical detection (ED) by either a LCA 15 EDT electrochemical detector (EDT Research, London, UK) or a 5100A Coulochem electrochemical detector, guard cell 5020 and analytical cell 5011 (loaned by Severn Analytical, Beds., UK). A reversed-phase C_{18} Nucleosil 300 Å, 7-μm analytical (250 mm × 4.9 mm I.D.) and guard (50 mm × 4.9 mm I.D.) columns (Hichrom, Berks., UK) were employed.

The standard mobile phase constituents comprised acetonitrile–0.1 *M* potassium dihydrogenphosphate adjusted to pH 2.3 with Aristar phosphoric acid (32:68), at a flow-rate of 1 ml/min. These conditions were varied systematically and are reported in the appropriate sections that follow. Samples were injected manually via a 50μl loop. Sample concentration varied between 5 and 10 μg/ml but was sufficiently small to avoid column overloading. Chromatograms were recorded and analysed with respect to peak height, peak area, retention time, capacity factor (k'), peak asymmetry factor (A_s) and the effective theoretical plate height equivalent (H_{eff}) was calculated for those peaks that could be adequately described as Gaussian [28].

RESULTS AND DISCUSSION

Effects of HPLC mobile phase constituents on the chromatographic behaviour of β-endorphin

Percent of acetonitrile. It was demonstrated that β-endorphin is extremely sensitive to solvent polarity. A narrow range of elution was exhibited for β-endorphin of between 30 and 34% acetonitrile, 29% retaining the molecule indefinitely on the column and 36% eluting it in the solvent front (Table I). Similar steep elution profiles for other polypeptides have been observed [5,29,30].

The effect of acetonitrile concentration on the elution of α- and γ-endorphin as well as for Met and Leu enkephalin were studied under similar conditions. Results are given in Table I.

The displacement model theory developed by

TABLE I

THE EFFECT OF VARYING THE PERCENTAGE ACETONITRILE ON k' VALUES FOR MET-ENKEPHALIN, LEU-ENKEPHALIN, α-ENDORPHIN, β-ENDORPHIN AND γ-ENDORPHIN

Acetonitrile (%)	k'				
	γ-Endorphin	α-Endorphin	β-Endorphin	Leu-enkephalin	Met-enkephalin
26	2.9	0.6	–	1.4	0.9
27	2.4	0.5	–	1.3	0.8
28	1.7	0.5	–	1.1	0.6
30	0.9	0.4	2.8	0.8	0.6
31	–	–	1.0	–	–
32	0.5	0.3	0.8	0.6	0.4
33	–	–	0.7	–	–
34	–	–	0.5	–	–
35	–	–	0.3	–	–

Geng and Regnier[31] can be used to estimate the relative contact areas between these solute molecules and the stationary phase due to the adsorption process. According to this theory (k') can be expressed as

$$\log k' = \log I - Z \log D$$

where D is the molar concentration of the organic modifier, Z is a measure of the contact area of the absorbed peptides and I represents a measure of the relative binding strength of individual polypeptides under a fixed mobile phase composition.

Linear relationships were obtained for plots of $\log k'$ as a function of $\log D$ for α-endorphin, γ-endorphin, Met-enkephalin and Leu-enkephalin (as shown by their correlation coefficients in Table II), the plot for β-endorphin was, however, curvilinear (Fig. 1). The values of Z for the above peptides are given in Table II, and that for β-endorphin was taken from the slope at 32% acetonitrile. Even though β-endorphin is nearly twice the molecular weight of γ-endorphin it has a slightly smaller contact area which would suggest that it is in a folded form under these conditions. Similarly, it can be argued that α-endorphin is folded having a contact area slightly smaller than the five amino-acid peptides, Leu- and Met-enkelphalin.

pH. The effect of changes in aqueous phase pH on the elution of β-endorphin was determined by HPLC–ED. A pH range of between 2.3–4.0 was attained with H_3PO_4, all other mobile phase constituents remained constant. Table III details alterations observed in the chromatographic performance of β-endorphin.

Peak area was shown to increase from pH 2.3–3.2 ($p < 0.05$), however, a dramatic and significant decrease ($p < 0.01$) occurred at pH 4.0 (Table 3). This observation supports the findings of others that a low pH of between 2.0–3.0 is required if maximum chromatographic efficiency of pro-opiomelanocortin-related peptides is to be obtained [4,32]. Identical k' values were gained between pH 2.3–3.2, however at pH 4.0 k' was significantly increased ($p < 0.01$). This suggests variation in the interaction of β-endorphin with the stationary phase, and is shown by the change in H_{eff}. While the β-endorphin molecule, having a p*I* at pH 9.9 [33], will be positively charged between pH 2.3–4.0, localised change

TABLE II

VALUES OF Z AND CORRELATION COEFFICIENTS (r) FOR A RANGE OF ENDOGENOUS OPIOIDS

	γ-Endorphin	α-Endorphin	Leu-enkephalin	Met-enkephalin	β-Endorphin
Z	8.8	3.2	4.5	4.1	7.2
r ($n=5$)	0.997	0.999	0.992	0.976	

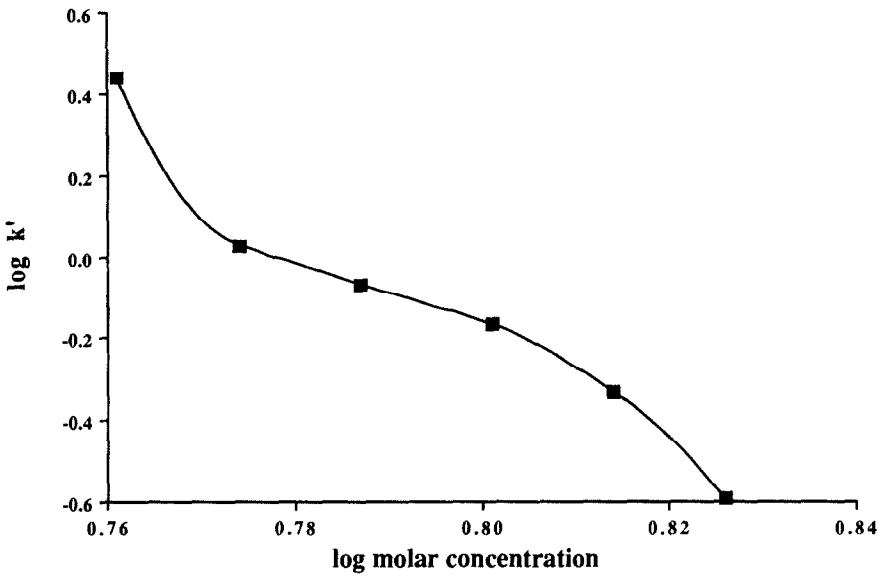

Fig. 1. Effect of mobile phase polarity on the chromatographic retention of β-endorphin: log k' for β-endorphin *versus* log molar concentration of acetonitrile.

in the ionisation of the acidic/carboxylic side chains of glutamic acid and the C-terminal may be possible at the higher end of this pH range. It is postulated, therefore, that the dramatic reduction in peak area, the change in k' and H_{eff} is the result of negatively charged regions of the molecule causing slight conformational change, possibly reducing the tyrosine accessibility and thus the signal generated and changing the interaction of the molecule with the stationary phase.

TABLE III

EFFECTS OF VARYING AQUEOUS PHASE pH ON THE CHROMATOGRAPHIC PERFORMANCE OF β-ENDORPHIN [MOBILE PHASE CH_3CN–0.1 *M* KH_2PO_4 (32:68), ACIDIFICATION GAINED WITH ARISTAR H_3PO_4]

	pH			
	2.3	2.8	3.2	4.0
Peak area (mm^2)	69	76	97[a]	49[b]
k'	1.4	1.4	1.4	2.1[b]
H_{eff} (μm)	472	472	472	200[b]

[a] Mann Witney "U" Test; $p < 0.05$ [39].
[b] Mann Witney "U" Test; $p < 0.01$.

Molarity. Effects of changes in molarity of KH_2PO_4 on the peak area of β-endorphin were investigated using EDT-ED. A constant pH of 2.3 was maintained throughout by adjustment with Aristar H_3PO_4. Results detailing peak areas as a function of molarity are illustrated in Table IV. A sharp peak area–molarity profile was demonstrated with the greatest peak area being recorded at 0.1 *M* KH_2PO_4, while peak resolution deteriorated above and below 0.1 *M* KH_2PO_4 (Table IV). The reduction in peak asymmetry noted from 0.01 to 0.1 *M* KH_2PO_4, pH 2.3 can perhaps be explained by a

TABLE IV

EFFECTS OF VARYING AQUEOUS PHASE MOLARITY (0.010–0.150 *M* KH_2PO_4) ON THE CHROMATOGRAPHIC PERFORMANCE OF β-ENDORPHIN [MOBILE PHASE CH_3CN–KH_2PO_4, pH 2.3 (32:68)]

Molarity	k'	A_s	Peak area (mm^2)
0.010	2.2	9.0	95
0.050	1.9	28.0	145
0.075	1.6	20.0	178
0.100	1.1	1.0	262
0.150	1.0	0.4	184

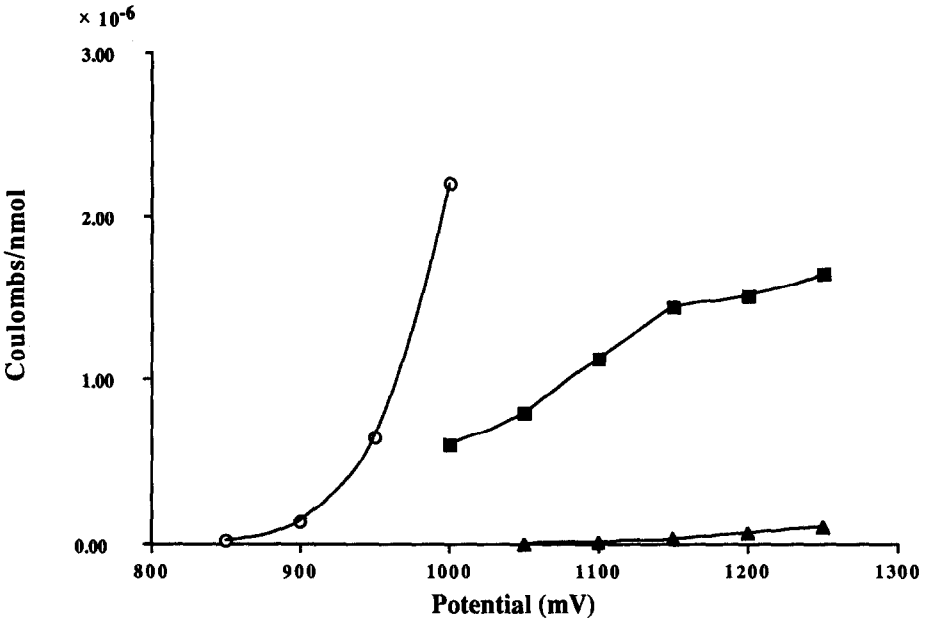

Fig. 2. Hydrodynamic voltammograms of β-endorphin (■), tyrosine (○) and methionine (▲) gained utilising a LCA 15 EDT electrochemical detector.

conformational change of β-endorphin from a more unstructured to a relatively more structured form. The higher buffer molarities (>0.1 *M*) required for β-endorphin chromatography, have been reported to be mandatory for reproducible high-efficiency chromatography of other polypeptides [33].

Comparison of the sensitivity to detection of tyrosine, methionine and β-endorphin. Hydrodynamic voltammograms constructed for β-endorphin and its two major electrochemically active amino acids, tyrosine and methionine are shown in Fig. 2. Tyrosine displays superior electrochemical activity to β-endorphin, suggesting that the activity of β-endorphin is not an additive function of electrochemical activity of the constituent amino acids. Methionine, conversely, would only be expected to contribute fractionally to the activity of β-endorphin. Although β-endorphin contains one methionine and two tyrosine residues within its structure it exhibits a dramatically lower sensitivity to oxidation per mole than tyrosine. Whilst some of this difference can be attributed to the lower mobility of β-endorphin, calculations (shown in Table V) indicate that another phenomenon is occurring.

The notion that β-endorphin exhibits α-helix conformation might be postulated as an appropriate explanation. This premise is supported by Lichtarge *et al.* [18] finding that in organic modifier β-endorphin undergoes conformation changes, inducing an α-helix structure between residues Tyr 1–Thu 12,

TABLE V

THE OBSERVED AND CALCULATED RATIO DIFFERENCE BETWEEN THE SIGNAL GENERATED FOR TYROSINE TO THAT OF β-ENDORPHIN AT VARYING POTENTIALS

The ratio difference between tyrosine and β-endorphin due to differences in diffusion coefficients (D_C) was calculated based on the assumption of spherical molecules where $D_C \propto 1/MW^{0.333}$. The two tyrosine residues present in β-endorphin have been considered in the calculation. MW = molecular weight.

Potential (mV)	Ratio difference	
	Observed	Calculated
1000	3.60	1.34
1050	5.25	1.34
1100	4.97	1.34
1150	4.27	1.34
1200	4.41	1.34
1250	4.36	1.34

Leu 14–Lys 28 and a β-turn between Lys 28–Glu 31. The tyrosine residues at positions 1 and 27 are thus an integral part of the changes in conformation which may result in the shielding of active amino acids and thus a loss of activity.

Environmentally induced conformational changes in β-endorphin

The effect of reconstituting β-endorphin in varying percentages of CH_3CN and KH_2PO_4 on HPLC chromatograms. Lyophilised samples of β-endorphin were reconstituted with varying percentages of CH_3CN and 0.1 *M* KH_2PO_4 adjusted to pH 2.3 with H_3PO_4 (v/v) to achieve a range of 0 to 50% acetonitrile. The final concentration of β-endorphin was 5 μg/ml (samples A) or 10 μg/ml (samples B) irrespective of the solvent composition. HPLC–UV analysis of β-endorphin at both 210 nm (samples A) and 275 nm (samples B) was then undertaken. This allowed the effect of sample solvent environment on the subsequent chromatography of β-endorphin to be established (Table VI).

A similar relationship is apparent for peak area at both wavelengths, an increase being observed from 0 to 30% CH_3CN but a decrease noted thereafter (Table VI). This can perhaps be attributed to the internalisation of the tyrosine and phenylalanine residues in β-endorphin, as at 275 nm the aromatic absorption of these amino acids has been dramatically reduced. At 38 and 40% CH_3CN concentrations, asymmetrical peaks appeared which had progressed to be either large shoulders or split peaks at 50% CH_3CN (*i.e.* Fig. 3). While the interaction of β-endorphin with the mobile and stationary phase did not change, denoted by a markedly constant k' value, the chromatographic efficiency was demonstrated to be increasingly jeopardised as the percentage of acetonitrile rose in the reconstitution fluid. It might be assumed therefore that when β-endorphin is injected onto the HPLC column in a solution containing higher concentrations of acetonitrile than that present in the mobile phase, that the conformational modifications which would be expected to result, as the molecule restructures to orientate itself in the mobile phase (*i.e.* 32% CH_3CN–0.1 *M* KH_2PO_4, pH 2.3), are significantly slower than its life time on the column.

To further verify the above observations and to establish whether conformational changes are reversible, additional analysis was undertaken. A sample of β-endorphin (10 μg/ml) was reconstituted and divided equally into two aliquots and diluted to make final concentrations of 5 μg/ml of β-endorphin in either CH_3CN–KH_2PO_4, pH 2.3 (30:70) (A) or CH_3CN–KH_2PO_4, pH 2.3 (50:50) (B). Each sample was assessed for its β-endorphin content using HPLC–UV (210 nm), peak areas and k' values were recorded. To check reversibility, sample B was further processed by altering the proportions of solvent present to that of (A); CH_3CN–KH_2PO_4, pH 2.3 (30:70) (C), and then re-analysed (Table VII).

It was demonstrated that the conformational change of β-endorphin, induced by variation in the proportions of CH_3CN and KH_2PO_4, is completely

TABLE VI

EFFECTS OF SAMPLE SOLVENT ENVIRONMENT ON THE SUBSEQUENT CHROMATOGRAPHIC PERFORMANCE OF β-ENDORPHIN

Lyophilised β-endorphin samples were reconstituted with varying percentage of CH_3CN to 0.1 *M* KH_2PO_4 pH 2.3 and chromatographically analysed utilising a mobile phase of CH_3CN–KH_2PO_4, pH 2.3 (32:68).

nm	Parameter	CH_3CN in the reconstitution solution (%)									
		0	10	20	25	30	32	34	36	38	40
210	Peak area (mm^2)	154.0	221.0	274.0	325.0	332.0	325.0	301.0	256.0	234.0	179.0
	±S.D. (mm^2)	11.0	26.0	16.0	5.0	9.5	24.0	28.5	25.5	8.5	1.5
	k'	0.9	0.9	0.9	0.9	0.9	0.9	1.0	1.0	0.9	0.9
275	Peak area (mm^2)	–	–	–	64.5	69.0	67.0	51.0	36.0	25.0	18.2
	±S.D. (mm^2)	–	–	–	1.0	2.5	3.0	2.0	2.5	2.0	1.5
	k'	–	–	–	0.8	0.9	0.8	0.9	0.9	0.8	0.8

[a] S.D. ± Standard deviation (n= 5).

Fig. 3. Reversed-phase chromatographic behaviour of β-endorphin as a result of changes in sample solvent composition prior to injection onto the HPLC column. Chromatograms are those which were gained when a sample of β-endorphin was reconstituted with either (A) CH_3CN–KH_2PO_4, pH 2.3 (40:60) or (B) CH_3CN–KH_2PO_4, pH 2.3 (50:50). The first and second eluting peaks are denoted by 1 and 2, respectively. Isocratic mobile phase constituents were CH_3CN–KH_2PO_4 (adjusted to pH 2.3 with Aristar H_3PO_4) (32:68).

reversible. The HPLC–UV response obtained for β-endorphin in solvent B, as measured by peak area and k', returns to the original values gained for solvent A. It is evident therefore that a slight alteration in the sample solvent environment and the subsequent chromatography of β-endorphin may be manifested as a major change in chromatographic performance.

The effect of HPLC flow-rate on β-endorphin chromatograms. Assessment of the chromatographic performance of certain proteins has been studied by varying the HPLC flow-rate [10,11,27]. This provides a method of altering the retention time of a molecule, thereby enabling a reversibly denatured protein to be eluted at varying stages during its transition back to its native form. An examination of the equilibration rate of β-endorphin was thus undertaken by manipulating the flow-rate and assessing the corresponding chromatograms. HPLC–UV conditions utilised were those routinely used, CH_3CN–0.1 *M* KH_2PO_4 adjusted to pH 2.3 with H_3PO_4 (32:68). β-Endorphin samples reconstituted in this mobile phase and in addition, varying percentages of CH_3CN and 0.1 *M* KH_2PO_4 adjusted to pH 2.3 with H_3PO_4 were subjected to changes in flow-rate. Each sample of β-endorphin was 5 μg/ml, with the same sample being injected at all investigatory flow-rates. The relationship between flow-rate, peak area, k', retention time and solvent composition is demonstrated in Table VIII. As might be expected, retention time increases with decreasing flow-rate, the relationship of the increase proving to be similar, reflected by the k' values, irrespective of the sample solvent concentration. Thus the nature of interaction between molecule, stationary and mobile phase is therefore, perhaps alike.

In support of findings documented earlier, the sample solvent environment prior to HPLC analysis was again shown to directly affect the chromatographic behaviour of β-endorphin by dramatically altering the size and shape of the peak. Furthermore, the samples' solvent environment was also demonstrated to influence changes in peak area with altered flow-rates.

A decrease in flow-rate resulted in an increase in peak area, this increase when expressed as a ratio of the peak area at 1 ml/min was shown to be the same

TABLE VII

REVERSIBLE CHANGES IN THE CHROMATOGRAPHIC PEAK AREA OF β-ENDORPHIN AS A CONSEQUENCE OF ALTERATIONS IN THE SAMPLE SOLVENT ENVIRONMENT PRIOR TO CHROMATOGRAPHY

Sample	Proportion of CH_3CN: KH_2PO_4	Peak area (mm^2)	k'	
A	30 : 70	328		0.8
B	50 : 50	108	0.7[a]	0.8
C	30 : 70	333		0.8

[a] Developing peak.

TABLE VIII

THE EFFECT OF FLOW-RATE ON THE CHROMATOGRAPHY OF β-ENDORPHIN RECONSTITUTED WITH VARYING PERCENTAGE OF CH_3CN TO 0.1 *M* KH_2PO_4, pH 2.3 AND ANALYSED UTILISING A MOBILE PHASE OF CH_3CN–0.1 *M* KH_2PO_4, pH 2.3 (32:68)

Flow-rate (ml/min)	Ratio difference	Reconstitution of β-endorphin (% acetonitrile)									
		32%					36%				
		Peak area	Ratio difference	Peak area · flow-rate	R.T.[a]	k'	Peak area	Ratio difference	Peak area · flow-rate	R.T.	k'
1.0	1.25	337	1.3	337	6.3	0.75	225	1.2	255	6.2	0.88
0.8	1.66	439	1.5	351	7.7	0.75	300	1.9	240	7.5	0.79
0.6	2.50	517	2.4	310	10.1	0.90	485	2.8	291	10.0	0.72
0.4		821		328	15.2	0.73	720		288	14.9	0.77
Flow-rate (ml/min)	Ratio difference	Reconstitution of β-endorphin (% acetonitrile)									
		40%					50%				
		Peak area	Ratio difference	Peak area · flow-rate	R.T.	k'	Peak area	Ratio difference	Peak area · flow-rate	R.T.	k'
1.0	1.25	204	1.2	204	6.5	0.80	110	1.1	110	6.6[b]	0.83
0.8	1.66	252	1.3	202	7.6	0.81	120	1.3	96	7.8[b]	0.86
0.6	2.50	275	1.6	165	10.1	0.80	147	1.4	88	10.2[b]	0.76
0.4		336		134	14.6	0.73	150		60	15.4[b]	0.75

[a] R.T. = Retention time.
[b] Developing peak.

as the ratio change in flow-rate for samples of β-endorphin in both the CH_3CN–KH_2PO_4 (32:68) and CH_3CN–KH_2PO_4 (36:64). In higher acetonitrile concentrations, however, ratio differences in peak area are much smaller (Table VIII) than might be anticipated. In addition, when the peak areas are multiplied by their respective flow-rate a constant value should be gained [28], and while the CH_3CN–KH_2PO_4 (32:68) and CH_3CN–KH_2PO_4 (36:64) peak areas do yield such a value, CH_3CN–KH_2PO_4 (40:60) and CH_3CN–KH_2PO_4 (50:50) do not (Table VIII). These inconsistencies at higher acetonitrile concentrations might be explained by the conformational state of β-endorphin.

The reduction in β-endorphin peak area noted at all flow-rates for both CH_3CN–KH_2PO_4 (40:60) and CH_3CN–KH_2PO_4 (50:50) is perhaps due to the molecule being in a more structured form. Thus, if this is true then the conformation of β-endorphin prior to analysis is crucial to its chromatographic performance.

Chromatographically induced changes in other proteins have been ascertained and are described by a two-state model [9,11] where the native and denatured peaks are inversely related. If β-endorphin is helical or partially helical in the mobile phase and is reversibly denatured by the stationary phase, then the molecule would be expected to fold to reform the native peak on its elution from the column, as has been shown for other proteins [9,10]. Such a phenomenon is, however, not apparent. Findings do suggest though that β-endorphin does have the propensity to alter structure when reconstituted and introduced onto the column in higher percentages of acetonitrile. In addition the occurrence of asymmetrical and split peaks provide evidence that a further confirmation of β-endorphin can exist and may be induced under certain conditions, although an adequate explanation of such phenomena cannot yet be given. Information obtained therefore, only highlights that the chromatography of β-endorphin involves a complex mechanism of separation, which is particularly sensitive to the micro-environment of the molecule and suggests that a more complex model as described for other proteins [8] is required to explain the dynamic effects of β-endorphin.

The effect of HPLC column temperature on β-endorphin chromatograms. To study further the chromatographically induced conformational change of β-endorphin, thermal denaturation was utilised. This technique has been used by other investigators in an attempt to characterise the denaturated and native forms of proteins [10,26,27]. The column temperature was thermostatically controlled by a

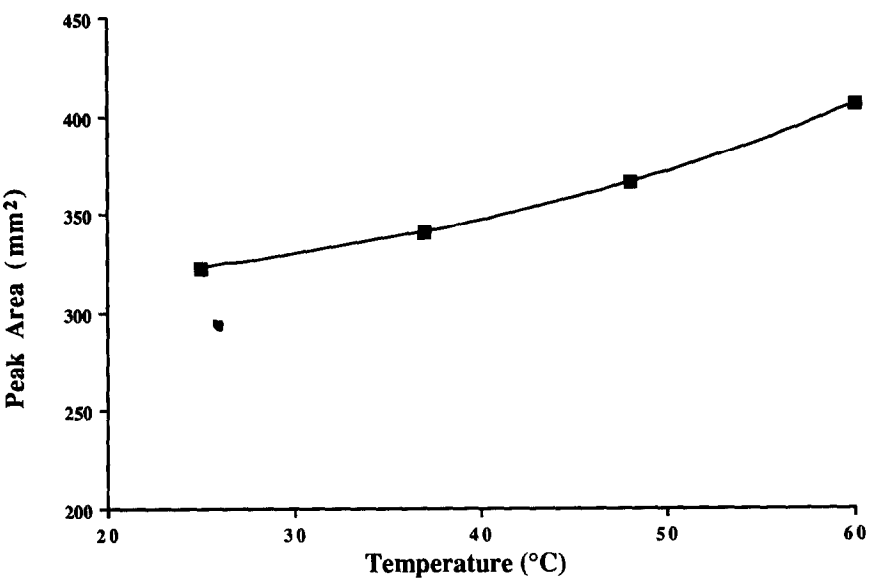

Fig. 4. The effect of elevating HPLC column temperature on the chromatography of β-endorphin, measured as changes in peak area (mm^2).

condenser. A thermocouple 4939 (Comark Electronics), was placed between the column and the jacket so that temperature could be accurately measured. The same sample of β-endorphin (5 μg/ml) was used at all temperatures; 25, 37, 48 and 60°C, so as to ensure that any difference observed in peak area could be ascribed to temperature alone, rather than changes in sample concentration. Results are demonstrated in Fig. 4, and show an increase in peak area with an increase in temperature. As temperature is known to destructure proteins, then it can be assumed that β-endorphin is transformed from a helical structure to a denatured random coil, thus altering the micro-environment of the absorbing amino acids resulting in an increase in response. These data support previous findings [13,14] that β-endorphin is helical during detection after undergoing HPLC at room temperature (25°C).

In addition to the increase in peak area caused by a rise in temperature, the introduction of a small peak was observed at 37°C which persisted up to 64°C where this peak began to split. Interestingly, these peaks eluted prior to the main peak. As it is thought that a denatured protein exposes additional internal hydrophobic residues, which increase its interaction with the stationary phase and thereby lengthen the retention time [27], then it might be supposed that these early eluting peaks represent a more folded form of the molecule than the main peak. The appearance of the split peak can perhaps only be explained by the emergence of another conformational form of β-endorphin.

Substantiating the hypothesis that the main peak of β-endorphin is unfolding, data gained demonstrates an increase in k' with elevated temperature (Table IX), which would normally be expected to decrease [34]. Elevated temperature will facilitate the denaturation of a peptide resulting in more sites being available to associate with the stationary phase, thereby lengthening the retention time. It might therefore be suggested that the main eluted peak, in this instance, represents an increasingly unfolded form of β-endorphin. These findings further demonstrate that the dynamic processes involved do not fit a simple two-state kinetic model.

TABLE IX

EFFECT OF COLUMN TEMPERATURE ON k' VALUES OF THE MAIN ELUTED PEAK OF β-ENDORPHIN [MOBILE PHASE UTILISED WAS CH_3CN–0.1 *M* KH_2PO_4, pH 2.3 (32:68)]

Temperature (°C)	k'		
	Peak 1	Peak 2	Peak 3
25			0.9
37		0.8	0.95
48		0.8	1.0
60		0.8	1.05
64	0.7	0.8	1.1

CONCLUSIONS

The observation that β-endorphin has the propensity to alter conformation during HPLC analysis [14,15] has been confirmed within this study. Mobile phase constituents, such as organic modifier, pH and buffer molarity dramatically alter the chromatographic behaviour of β-endorphin. The exact mechanisms involved cannot be predicted from the present work, although it would seem that the interaction of the molecule is not just reliant upon the sorbent and mobile phase but also the structure of β-endorphin prior to injection onto the column.

Conformational change of proteins normally occurs over a time span of 0.1 to 100 S [35]. Thus depending on the rate of equilibration between the native and denatured protein, whether it is possible to study the HPLC-induced conformational transition by manipulating the flow-rate. If this conformational process is rapid one peak will appear on the chromatogram, however, two or more peaks will be evident if the rate is slow [36]. Results gained can only partially be explained by the above, as while an extra peak did appear at slower flow-rates it was evident only as a consequence of variation in the micro-environment of the sample prior to chromatography. It is apparent therefore that the conformational change of β-endorphin, in this instant, cannot be adequately explained by the two-state model of kinetic processes.

The way in which β-endorphin folds is unknown, although some of the general assumptions forwarded for proteins [35] may apply here. In addition, as β-endorphin contains a proline at residue 13 it is possible that *cis–trans* isomerisation occurs, promoting conformational change of this peptide. This

phenomenon has altered the chromatographic performance of other peptides causing band spreading and peak distortion [36–38].

From this work it can be concluded that β-endorphin can undergo conformational changes during HPLC and that the sample solvent prior to injection can dramatically alter the chromatogram.

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